STUDIES OF *Lutzomyia anthophora* (ADDIS) (DIPTERA: PSYCHODIDAE) AND OTHER POTENTIAL VECTORS OF RIO GRANDE VIRUS

By

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A man's reach should exceed his grasp.

--Robert Browning

Lutzomyia anthophora feeding on the ear of its native host, the woodrat, *Neotoma micropus*.

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The question of how to thank someone for his years of friendship, guidance, and wise counsel remains enigmatic. During the course of this program I have incurred many debts which I will hopefully repay through contributions to the science.

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Chairman: Dr. Harvey Cromroy
Major Department: Entomology and Nematology

Simple colonization techniques for rearing large numbers of phlebotomine sandflies were developed. Lutzomyia anthophora (Addis) and Lutzomyia diabolica (Hall) were colonized in the laboratory for the first time for 16+ and 7+ generations, respectively, thus permitting quantitative investigation of their ability to transmit viruses and leishmaniasis. Notes on field behavior of L. anthophora and L. diabolica are presented with detailed laboratory studies on the biology of the two species. Laboratory transovarian transmission of a Phlebovirus was demonstrated for the first time with L. anthophora when 54.8% of the F_1 adult progeny from parents infected by intrathoracic inoculation became infected. Attempts to transmit Rio Grande virus by the bite of L. anthophora and Triatoma gerstaeckeri (Stal) were unsuccessful.

GENERAL RATIONALE

The primary goal of this research was to conclusively demonstrate transovarian transmission of a Phlebovirus in a sandfly for the first time. A virus (Rio Grande), non-pathogenic for humans, was selected because it could be safely studied. The potential vector sandfly, Lutzomyia anthophora, is not anthropophilic and therefore is a safe subject for study.

Before transmission experiments could be undertaken it was necessary to first develop sandfly rearing and colonization techniques. In order to plan and execute transmission experiments some aspects of the laboratory biology of L. anthophora had to be elucidated.

After transovarian transmission was demonstrated, I realized that this alone could not account for the distribution of neutralizing antibody in various animals from south Texas in view of the fact that L. anthophora apparently does not feed on all of them. In order to more fully understand the ecology of Rio Grande virus, preliminary studies of other hematophagous insects, L. diabolica and Triatoma gerstaeckeri, were undertaken.

In a broad sense it must be acknowledged that no single mechanism such as transovarian transmission can account for the maintenance of a virus when the species of interest is sympatric with other hematophages. Each insect species that feeds on the host must be studied to determine its relative role in the maintenance of a pathogen.

SECTION I

TECHNIQUES FOR LABORATORY REARING OF SANDFLIES (DIPTERA: PSYCHODIDAE)

Introduction and Literature Review

The difficulty of efficiently producing large numbers of sandflies in the laboratory has hindered studies on their biology and vector competence for viral and parasitic diseases (Killick-Kendrick 1978). Despite significant contributions by several workers (Chaniotis 1967, 1975, Gemetchu 1976, Killick-Kendrick et al. 1973, Killick-Kendrick et al. 1977, Ward 1977) several major problems remain. Some of these include (1) larval mortality due to unknown factors, (2) excessive labor requirements for colony maintenance, and (3) death of females at oviposition. Use of the techniques described here have considerably reduced the first two difficulties and partly solved the third.

Six of the 600 known phlebotomine species, e.g. P. argentipes Annandale & Brunetti, P. papatasi (Scopoli), L. longipalpis (Lutz & Neiva), L. sanguinaria (Fairchild & Hertig), L. gomezi (Nitzulescu), and L. flaviscutellata (Mangabeira), have been reared for 10 generations or more (Killick-Kendrick 1978, Ward 1977). The following species have been reared by the methods described here: L. anthophora (Addis), 15 generations; L. shannoni (Dyar), 15 generations; L. vexator vexator (Coquillett), 7 generations; L. diabolica (Hall), 5 generations;

L. cruciata spp, 23 generations; L. cayennensis (Floch & Abonnenc), 5 generations. In addition, 3 African phlebotomine species were reared to the 4th generation using these methods (D. Young, personal communication).

General Techniques

Figure 1-1 represents the generalized rearing method for sandflies. An explanation of each step is as follows: Step 1. The plaster of Paris in a rearing cage is saturated with H_2O with no free water remaining; Step 2. An engorged female is gently "herded" into the vial. A drop of 30% honey solution or other sugar source is then placed on the screen top; Step 3. After 3+ days most females oviposit on the plaster bottom. If the female survived oviposition she is released back into the feeding cage. Screen lids are replaced with solid tops that have small punctures to allow for gas exchange but limit dessication; Step 4. Since eggs held at 26°C usually hatch 6-14 days after oviposition, a small amount of larval diet is placed in the vial 4-5 days after the eggs are laid; Step 5. Larvae should be checked weekly and moist medium added as required; Step 6. Adults are released into the feeding cage daily by placing lidless vials containing pupae in the feeding cage. Adults soon begin mating and feeding on sugar from apple slices provided; Step 7. An anesthetized or restrained vertebrate host is placed inside the cage after a prefeeding period that varies in time according to species.

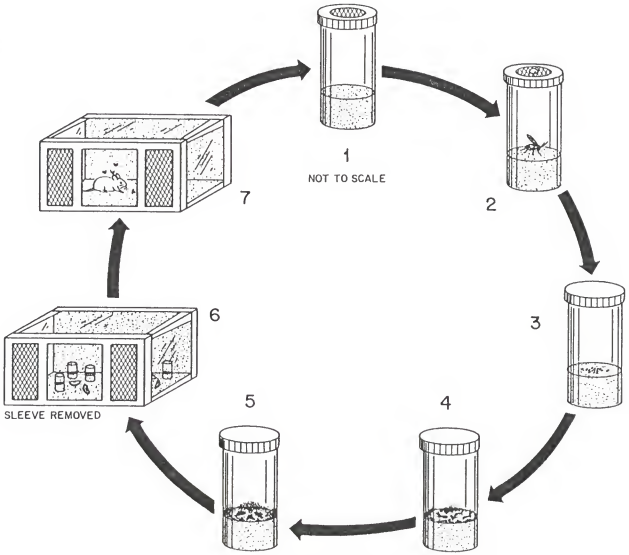


Figure 1-1. Schematic diagram of laboratory rearing techniques for phlebotomine sandflies.

Larval Rearing

Later instar larvae are more tolerant of moisture variation than earlier instar larvae. When larval medium (Young et al. 1981) is added to the rearing vials it must be slightly moistened. Larvae can be reared under conditions of >80% RH but 90-95% is preferable. Even at this high humidity secondary fungal growth is uncommon, presumably because the first Rhizopus sp. bloom either exhausts an essential nutrient or produces a fungal growth inhibitor. It is a primary colonist in fungal succession and reduces proteins to amino acids and carbohydrates to simple sugars. After the medium has completely dried, it is remoistened. Even then, there is little fungal growth, the hyphae are not abundant enough to entangle the caudal setae of the larvae.

Mites frequently seen in larval vials have not been observed attacking healthy larvae but they will feed on weak or dead larvae and adults. Boiling water poured into vials before reuse will kill any mites present. Autoclaving larval medium infested by mites for 5 min at 15 psi will kill mites without apparent damage to the medium.

Sugar Feeding

Ready (1979) provided evidence that sugar feeding is important for sandfly egg production. Adults were provided sugar ad libidum throughout their lifetime by two methods.

Adult males and non-bloodfed females were provided sugar from thin apple slices (<3 mm) leaned against the sides of the feeding cages (Figure 1-2). Thicker apple slices tend to mold more rapidly than

Figure 1-2. L. anthophora feeding on an apple slice.

thin ones which tend to dry. A tangential section of each apple slice should be removed to produce a flat edge so that the slice will not roll and crush flies. Fresh slices are added daily. Rhizopus sp. is the mold that usually grows on "old" apple slices and it can be used as inoculum for larval medium.

Bloodfed females are provided sugar in the oviposition cages by placing a small drop of 30% honey solution or 50% Karo syrup® solution on the screen lid. A 30% honey solution was used initially in an effort to produce a facsimile of nectar but a Karo syrup solution provided equivalent results. If fungal or bacterial growth become apparent in the sugar solution the lid should be changed and a new drop added. Refrigeration of stock sugar solutions at 3°C greatly increases their shelf life.

N-butyl Pthalate

Clear vinyl suction cups were used to suspend apple slices from the top of the feeding cage. This practice was quickly abandoned after adult mortality approaching 100% was associated with the vinyl use. N-butyl pthalate, an elasticizer used in vinyl, is volatile at room temperature and is highly toxic to sandflies and mosquitoes (David Carlson, biochemist, personal communication). The compound has been used as an insect repellant (The Merck Index 1976).

Adult Feeding

Two general methods for feeding adult females on an animals were used. The first method is to hold a 7 dram vial or 120 ml specimen

container of flies against an animal's ear or nose allowing the flies to feed through the screen top. This method is particularly useful for feeding flies on leishmanial lesions. A mesh size of 18/cm is required for small species such as L. anthophora and L. diabolica. A larger mesh size of 10/cm is adequate to contain larger species such as L. shannoni.

The second method is to place an anesthetized or restrained animal in the feeding cage. Anesthesia dosage rates are given in Table 1-1. Anesthesia was administered with a 1 ml Tuberculin syringe and a 26 or 27 gauge needle. Animals in poor condition require less anesthesia. An insufficient dose will sometimes produce hyperactive behavior.

Lid Cleaning

Screen tops that have been used for sugar feeding are cleaned by soaking in 5% Chlorox® solution for 30 min, rinsing 2x in tap water for 30 min, and air dried. Tops can be reused many times until screening material breaks or glue becomes brittle and non-adhesive. Use of a more concentrated Chlorox solution or a longer soaking time results in rapid deterioration of screen material and glue greatly reducing the number of times lids can be reused.

Adult Feeding Cages

The most successful adult feeding cages developed were constructed from 4 (26 x 20.5 x 16.5 cm), 6 (31.0 x 20.5 x 16.5 cm), and 12 (36 x 25.5 x 21.5 cm) liter aquariums (Figure 1-3).

Table 1-1. Dosage required to anesthetize animals for 30-60 min with Ketamine hydrochloride (100 mg/ml) injected IM.

Animal	Dosage (ml)
Mouse	0.05/adult
Squirrel	0.1/200 gm
Woodrat	0.2/adult
Opossum	0.25-0.30/2 kg
Rabbit	0.3-0.4 mg/kg

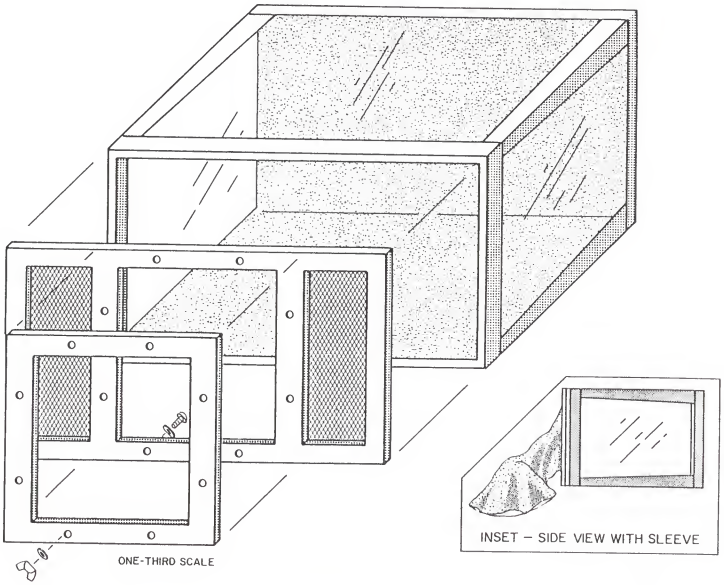


Figure 1-3. Sandfly feeding cage--A modified aquarium with plaster of Paris bottom and back.

The bottom and one side of the aquarium were covered with a 1 cm layer of plaster of Paris. After the bottom has been poured and allowed to dry it is imperative that it be saturated with water before the side layer of plaster is poured. This will prevent the formation of unworkable lumps at the junction of the two pours due to immediate dessication of the wet plaster by the dry layer. After the bottom and one side have been poured the bottom should be rewet and the upper corners filled in to a maximum depth of 2 cm. This allows for easy viewing of flies and easy recovery of flies with an aspirator.

Front panels for the cages are constructed of 64 mm (1/4") Plexiglas®. Screens of 18 mesh/cm of "Saran" (Chickopee Co., Cornelia, GA) vinylidene polymer plastic are installed on the front panel. Experience has shown that this is necessary because otherwise, excessive condensation in the chamber will form when animals are left in for sandfly feeding. The Saran screen is attached with epoxy cement. Care must be exercised that epoxy components are not out of date and are well mixed; otherwise the glue will remain sticky and trap the flies. Other glues tried, i.e., contact cement, Elmer's glue®, silicone, and superglue®, do not adhere well to the Plexiglas. The minimum screen areas are 78, 130, and 214 cm^2, respectively.

A 50 cm sleeve of 15.3 cm (6") surgical stockinet (Johnson & Johnson) is attached to the front panel by compression between the panel and a Plexiglas frame (2.5 cm wide). This is secured with 8 (10/24 x 1") brass screws with flat washers and wingnuts. The brass screws and flat washers are glued inside the front panel with epoxy glue in order to facilitate changing of the sleeve which should be secured with tape while the frame is being installed. The completed front panel is

attached to the cage with a thick layer of silicone glue that can be easily cut away for repairs. It is essential to fill all small crevices with plaster of Paris or silicone to prevent adult sandflies from hiding there and being difficult to recover.

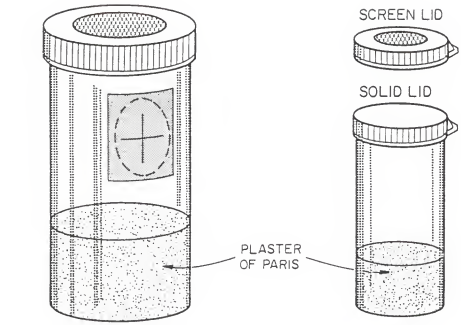
A cylindrical adult feeding cage (Figure 1-4) was constructed from a cylindrical (23 x 13 cm) glass fixture cover (Appleton Co. V-51). Four centimeters of plaster of Paris were poured in the end and 1.5 cm (tapered to the front) were poured on a side of cylinder by the method described. The frame was constructed of 3 (18 cm x 18 cm x 64 mm) Plexiglas plates and 18 cm (10/24) threaded steel rod. Relative positions of the plates is maintained by placing nuts and washers on both sides of the sheets which are attached to the glass by a bead of silicone glue. A 50 cm stockinet sleeve is secured to the front by compression between 2 plates as with the rectangular feeding chamber. The advantages of the cage include small size and ease of manufacture. Disadvantages are difficulty seeing through the glass, condensation due to animal respiration, and difficulty in manipulating vials inside the chamber.

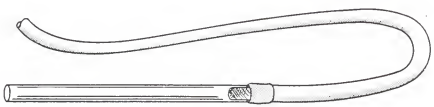
Field Collection, Feeding Containers

The 120 ml specimen containers (Pharmaseal Laboratories, Glendale, CA 91201) are modified for use as field collection and feeding containers (Figure 1-5).

Field collection containers are constructed as follows. Two centimeters of plaster of Paris are poured in the bottom of the containers; then a 2 cm entry port is cut in the container side by heating a #15 cork borer then pushing it through the plastic

Figure 1-4. Cylindrical adult feeding cage.





ASPIRATOR

ONE-THIRD SCALE

Figure 1-5. Field collection apparatus, feeding and rearing containers for phlebotomine sandflies.

which should be done in a well ventilated area to avoid noxious fumes. The edges of the hole are then filed smooth and pieces of latex surgical glove are glued to both sides with contact cement. If prepowdered surgical gloves are used they must be washed in a 70% ethanol solution to remove the powder to insure adhesion. Perpendicular cuts are made in the respective latex pieces producing a fly-proof opening for the insertion of an aspirator. Screen lids should be used on the containers when used for collecting vials. These containers can also be used for feeding flies. Screen lids are prepared by cutting a 4 cm hole in the plastic top and attaching the desired mesh screen with contact cement.

Individual Oviposition and Rearing Containers

These containers are made by pouring 1 cm of plaster of Paris in the bottom of a 7 dram plastic snap cap vial (Fisher Scientific Co., Pittsburgh, PA). When used for rearing containers the plastic tops are punctured to facilitate limited gas exchange. When used as feeding containers the lids are cut out with a #12 cork borer (1.5 cm hole) and covered with the desired mesh screen that is secured with contact cement. When the vials are inverted the plaster occasionally slides down crushing the insects. This can be prevented by pushing a hot pin through the plastic into the plaster then cutting off the excess.

Aspirators

Aspirators for collection and transfer of adults are constructed of thick wall latex tubing (10 mm ID x 15 mm OD x 60 cm) and thickwall Pyrex® glass tubing (12 mm ID x 15 mm OD x 30 cm) (Figure 1-5). The

latex tubing and glass are attached by a piece of hard plastic tubing (9 mm OD x 5 cm) covered with nylon organdy cloth on one end and secured with contact cement (Roberts Consolidated Industries, City of Industry, CA). The screened end is inserted into the glass tubing where it is held by friction and the latex tubing is pushed over the other end. The latex/glass junction is securely taped so that the end of the plastic tube is visible in the glass tubing.

Thickwall latex tubing is used for flexibility and to prevent kinks from occluding the passageway. Pyrex glass is used instead of plastic because plastic scratches easily making identification of specimens difficult. The inside diameter of any aspirator used for phlebotomine sandflies should be at least 10 mm because smaller diameters at the same suction pressure result in much higher intake velocities that cause damage to the flies.

SECTION II

COLONIZATION AND LABORATORY BIOLOGY OF THE SANDFLY, Lutzomyia anthophora (ADDIS) (DIPTERA: PSYCHODIDAE)

Introduction and Literature Review

Lutzomyia anthophora was first collected while feeding on rabbits in Uvalde, Uvalde Co., Texas (Addis 1945a). Subsequently it was reported from NE Mexico (Fairchild and Hertig 1956), SW Mexico (Vargas 1952), and SE Texas (Young 1972). Young (1972) reported finding L. anthophora in the nest of the plains woodrat, Neotoma micropus, with which it appeared to enjoy a close host-parasite relationship. Calisher et al. (1977) again reported the association of L. anthophora and Neotoma nests when suggesting that Rio Grande virus could be maintained in the woodrat population by transovarian transmission in this sandfly.

Addis (1945b) described the immature stages and the life cycle of L. anthophora after rearing 72 flies from egg-adult at 28-29°C. In this section detailed investigations of the colonization and biology of L. anthophora are reported.

Field Collections

Sandflies used to start the colony were collected by R.G. Endris and D.G. Young with the assistance of G.B. Fairchild and R.N. Johnson in the area of E and NE of Brownsville, Texas, from Neotoma nests in

May and June 1980. Vegetation was characterized by grasses, low growing shrubs, mesquite, and acacia trees common to xeric regions (Figure 2-1). Climatic conditions were quite dry at the time of collection; however, a rainy season occurs in August and September.

Johnson (1966) described the structure of woodrat nests in detail. The nests (Figure 2-2) were carefully disassembled and sandflies were collected with tube aspirators when seen hopping on the sticks. Flies were also recovered from under boards covering rodent burrows in a refuse dump. In both sites the soil was powder dry and the flies' moisture source remains an enigma. Aspirators and field collection containers have been described in Section I as well as methods for feeding freshly caught flies on hamsters.

As woodrats attempted to escape from their nests they were captured as a blood source for flies. Other woodrats and white-footed mice, Peromyscus leucopus, which also occupy woodrat dens, were trapped in Sherman traps.

Immature Behavior and Development

The rearing methods for establishing and maintaining the colony are described in Section I and by Young et al. (1981). Johnson and Hertig (1961) and Hanson (1968) grouped Neotropical phlebotomine larvae into two behavioral groups, i.e., those that burrow into the larval medium and those that are surface feeders. This behavior indicates where larvae may be found in nature, i.e., on the soil surface or burrowing beneath it. In the laboratory L. anthophora larvae exhibited no distinct behavioral preference and the degree of larval

Figure 2-1. Habitat of Lutzomyia anthophora.

Figure 2-2. Nest of Neotoma micropus.

burrowing appeared dependent on moisture content of the medium and the stage of development.

Larval emergence, behavior, and pupation were consistent with the observations of Chaniotis (1967), Johnson and Hertig (1961), and Gemetchu (1976). In contrast to the observation of Killick-Kendrick et al. (1977) with L. longipalpis, no cannabalism was observed among 4th instar larvae when starved. No effort was made to discover the larval habitat in nature although it is presumably in or under the woodrat nest (Young 1972).

Larval development rates at 4 different temperatures (20°C, 24°C, 28°C, and 32°C) were determined by rearing individual larvae in wells of microtitre plates that were 1/3 filled with plaster of Paris. Initially microtitre plates with 96 wells were used but the wells proved too small for 4th instar larvae. Twenty-four well microtitre plates were satisfactory. Attempts to use the lids designed for the multiwell plates were not successful because they do not seal well and larvae moved between wells. Lids (9 cm x 13 cm x 5 mm) made from Plexiglas and secured with elastic bands solved this problem (Figure 2-3). After several days in chambers at 90% relative humidity (RH) it was necessary to add drops of H_2O in each well until the plaster appeared damp.

Development times for immatures are presented in Table 2-1. The difference between the results of Addis (1945b) shown in Table 2-1 and those obtained in this study at 28°C are probably attributable to differences in larval diet.

Three larval diets at 28°C and two at 20°C were compared to determine the effect of diet on immature development time (Table 2-2). The diet prepared with Purina Rabbit Chow #5315 is that reported by Young et al. (1981).

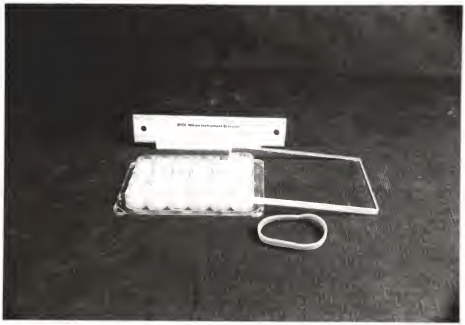


Figure 2-3. Multiwell plate with lid used for rearing individual larvae.

Table 2-1. Mean duration (days) of immature stages of L. anthophora at 90% RH and 4 constant temperature regimes: 20°C, 24°C, 28°C, and 32°C in contrast to the observations of Addis (1945b) made at 28-29°C. Larvae reared on the diet of Young et al. (1981).

Temperature (°C)	Egg	Larval Instars				Pupa	Total (egg-adult)
		1	2	3	4		
20	15.6±0.5 (59)*	12.7±3.3 (52)	8.5±2.5 (47)	9.9±4.7 (42)	22.0±6.9 (35)	16.8±3.3 (19)	83.3±9.3 (19)
24	10.0±0.6 (174)	6.3±0.8 (165)	5.0±0.8 (162)	5.2±0.3 (160)	11.3±1.0 (133)	11.5±0.4 (130)	49.5±2.0 (130)
28	8.0±0.1 (41)	3.9±0.8 (39)	3.1±1.1 (39)	8.3±1.0 (39)	8.7±2.2 (36)	8.0±0.8 (36)	36.1±2.9 (36)
28-29 (Addis)	10.5 (72)	28.4				8.7	49
32	6.7±0.6 (104)	3.7±1.3 (93)	3.8±0.8 (92)	3.9±1.2 (92)	8.8±2.3 (90)	6.7±0.8 (88)	33.5±3.7 (88)

*( ) indicates number of individuals surviving each stage.

Table 2-2. Comparison of the effect of larval diet composition prepared by the method of Young et al. (1981) on mean duration of immature development time (egg-adult) of L. anthophora at 20°C and 28°C, 90% RH.

Temperature (°C)	Diet Component		
	Rabbit Chow Purina #5315	Horse Chow Purina #3501	Laboratory Chow Number (?)
20	83.3±9.3 n = 19	99.6±6.0 n = 18	
28	36.1±2.9 n = 36	38.5±4.2 n = 34	43.5±4.1 n = 60

Time of Eclosion

It is a general observation that the males of many insect species begin eclosion before the females in order to be reproductively mature when the females emerge. I noted this to be the case with L. anthophora (Table 2-3), because mean development time from egg-adult was about 2 days less for males than females at temperatures above 20°C. This is noteworthy because males are not reproductively competent until 24 hrs post eclosion.

In order to demonstrate this phenomenon the sex and time of eclosion for all individuals from a cohort of the F_3 generation were recorded. The eclosion distribution of males and females is presented in Figure 2-4 and demonstrates the veracity of this observation. Sex ratios were 1:1.

Mating

Male genitalia rotate (Figure 2-5) about 12-24 hrs post eclosion after which they were observed mating. Females were seen mating within hours after eclosion and before, after, and during feeding. Mating frequency was not determined for either sex although males do mate more than once per lifetime. Copulation occurred regardless of nutritional state or photoperiod.

L. anthophora males demonstrated the "characteristic epigamic pattern" described by Chaniotis (1967). Mating usually lasted 2-5 min. Based on the criterion of egg fertility more than 85% of the females that laid eggs had successfully mated. In two generations studied the percentage of females laying infertile eggs was 13.7% and 16.7% in the F_8 and the F_{15} generations, respectively.

Table 2-3. L. anthophora--Comparison of mean development time (days) for males and females reared at 20°, 24°C, 28°C, and 32°C, 90% RH.

Sex	Temperature (°C)			
	20	24	28	32
Female	83.3±7.0 n = 10	50.7±3.6 n = 66	40.6±3.7 n = 73	34.6±4.0 n = 36
Male	83.3±11.7 n = 9	48.2±4.1 n = 63	37.5±3.3 n = 57	32.7±3.3 n = 52
Difference	0.0	2.5	3.1	1.9

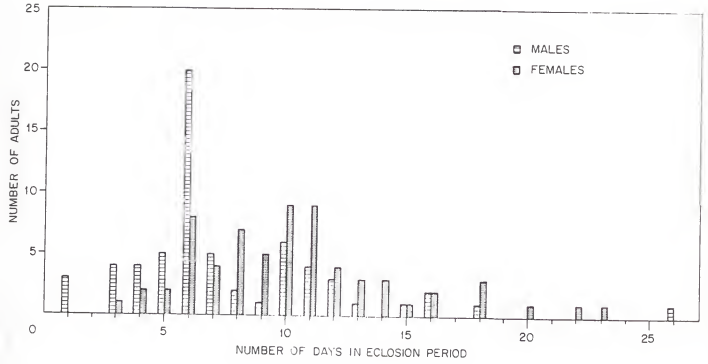


Figure 2-4. Eclosion distribution of 127 L. anthophora males and females from F3 generation in a laboratory colony at 24°C, 90% RH.

Figure 2-5. L. anthophora male <24 hrs old with unrotated genitalia.

Female Age at First Feeding

To determine when L. anthophora females were physiologically ready to take a blood meal, all adults from the F_{10} generation that eclosed each day were held in the cylindrical feeding chamber. Each day an anesthetized mouse was placed in the chamber for 60 min until all the females in each group either fed or died. Of 244 females, 92 (60.5%) fed within 1-7 days. The mean age at feeding was 3.7 days and the median age was 3.5 days. Addis (1945b) noted that females fed 2-4 days post eclosion. The temporal distribution of female age at feeding and the age of death for those flies that did not feed is presented in Figure (2-6).

The sugar feeding habits of hematophagous diptera are well known and most previous attempts to colonize sandflies included the provision of 30% sucrose solutions for the adults. Chaniotis (1974) and Ready (1979) investigated sugar feeding in phlebotomine sandflies. Chaniotis (1974) studied sandfly preference for various sugars and determined that sugar concentration had no effect on fly longevity. Ready (1979) found higher egg production among bloodfed females fed on sucrose solution vs. those fed on water. Killick-Kendrick (1979) suggested that the presence or absence of sugar in the gut may have profound and largely undetermined effects on the ability of sandflies to transmit leishmaniasis.

An experiment was conducted to determine what percentage of adults will ingest honey solutions, at what age, frequency of sugar feeding, and the time required to digest each meal. Individual pupae were placed in 7 dram feeding vials. The day of eclosion a small drop of 30% honey

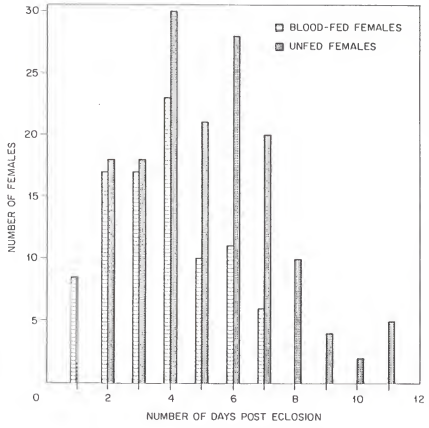


Figure 2-6. L. anthophora--Temporal age distribution at feeding and death of unfed females at 28°C, 90% RH.

solution mixed with either red, blue, or green food dye (C.F. Sauer Co., Richmond, VA) was placed on the screen lid. After a sandfly ingested the solution the dye was clearly discernible in the distended crop with or without the aid of a microscope (Figure 2-7). Yellow dye was not used because it could not be seen. When an individual digested the sugar solution a small colored droplet was excreted and no color remained in the abdomen. Flies would not refeed until the previous meal had been completely digested.

Results for sugar feeding experiments conducted at 20°C and 28°C are shown in Table 2-4. The reduction in number of flies feeding more than 1x at 28°C indicates the release of individuals back into the breeding colony rather than mortality. Of the flies offered honey solution at 28°C, 93.2% (68/73) fed within 24 hrs after eclosion. Of 23 flies held until death at 28°C, 100% fed 1x, 30.4% fed 2x, 30.4% fed 3x, 34.5% fed 4x, and 4% fed 5x.

The number of sugar fed adults held at 20°C declined rapidly due to poor survival. Digestion of the first and second sugar meal at 20°C requires more time than at 28°C.

Results of a second experiment to determine the effect of sugar feeding on percentage of females feeding on blood, fecundity, preoviposition period, mating (fertility of eggs), and mortality factors are presented in Table 2-5. Sugar feeding enhanced productivity for all parameters measured.

The source of sugars for L. anthophora in nature is unknown. Many woodrat nests are located around clumps of prickly-pear cactus (Opuntia lindheimeri), a succulent that woodrats feed on in their nests. Sandflies may obtain sugar from partly eaten cactus in the woodrat nest. To test

Figure 2-7. L. anthophora males and females engorged on 30% honey solution dyed with red, blue, and green food dye.

Table 2-4. L. anthophora--Adult sugar feeding, frequency, age of feeding (days), time required for digestion (days) at 20°C and 28°C, 90% RH.

Temperature (°C)	Age at 1° Feed	# Days to Digest 1° Meal	# Days to 2° Feed	# Days to Digest 2° Meal	# Days to 3° Feed	# Days to Digest 3° Meal	# Days to 4° Feed	# Days to Digest 4° Meal	# Days to 5° Feed
28°	1.3±0.8 n = 68	1.4±0.8 n = 48	1.4±1.1 n = 29	1.3±0.5 n = 25	1.5±0.7 n = 17	1.6±0.9 n = 16	1.8±1.1 n = 10	1.3±0.5 n = 6	1.0 n = 1
20°	1.9±1.0 n = 17	2.2±1.2 n = 10	1.2±0.5 n = 8	1.8±0.8 n = 6	1.2±0.4 n = 2	1.0 n = 2	1.0 n = 1		

Table 2-5. Comparison of effects of blood vs. blood and sugar as an energy source for L. anthophora fed on Didelphis marsupialis (opossum).

Energy Source	% Females Fed on Blood	% Bloodfed Females Laying No Eggs	Mean Number Eggs/Female	Preoviposition Period (Days)	% Fertility of Females Laying Eggs	% Females Peritrophic Sac Rupture	% Females Fed on Serum
Blood	40.8 (147)*	39.6 (60)	17.6±8.4	4.5±1.2	65.2	11.7	1.7
Blood + Sugar	49.7 (282)	19.8 (116)	28.0±13.2	6.2±1.8	83.3	4.9	0.7

*( ) indicates number in sample.

this hypothesis a piece of Opuntia was sliced and placed in a feeding cage with L. anthophora. Specimens were observed feeding on the plant juice but not as avidly as on apple slices.

Feeding Hosts

L. anthophora has been reported to feed on rodents and lagomorphs (Addis 1945a, Young 1972, and Calisher 1977). L. anthophora fed readily on the following anesthetized animals introduced into the feeding cage: Neotoma micropus (woodrat; Figure 2-7), Peromyscus leucopus (white-footed mouse), Mesocricetus auretus (Syrian hamster), Sciurus carolinensis (grey squirrel), Mus musculus (white mouse; Figure 2-8), Cavia porcellus (guinea pig), Oryctolagus cuniculus (domestic rabbit), and Didelphis marsupialis (opossum). The preferred feeding site was the nearly hairless portions of the ears. Fewer than 5% of the sandflies fed on the feet or among the vibrissae on the nose.

It is notable that L. anthophora would not feed on suckling mice when restrained with a cloth net on a tongue depressor. Attempts to feed the sandflies on the poikilotherms, Gopherus polyphemus (gopher tortoise) and Anolis carolinensis (anole), were unsuccessful.

Flies held in feeding cages on the ears of Canis familiaris (dog), and Ovis aries (sheep), did not feed. Efforts to feed the flies on Homo sapiens (human) were not successful. L. anthophora did feed on the ear of a 2 day old calf (Bos taurus) and on a shaved chick (Gallus gallus) restrained in a feeding cage.

When the sandflies did not feed on the preferred host they were subsequently offered either an opossum, woodrat, hamster, or mouse.

This was done to verify that the imagos were ready to feed. In each instance flies that refused the first host fed upon the second host.

It is apparent from the various species of mammals fed on that L. anthophora is a more opportunistic feeder than was previously recognized.

Feeding: Temperature Preference

On each of the host animals used several body regions were relatively hairless, i.e., the ears, nose, tail, and feet. To determine if relative temperature of the body regions influenced sandfly feeding site preference the skin temperature of these areas was measured with a BAT-4 Laboratory Thermometer (Bailey Instrument Co.) and a thermistor (Table 2-6). The instrument was calibrated to human body temperature of 37.2-37.4°C.

More than 95% of the sandflies fed on the ears where the skin temperature range was from 27.7°C to 37.8°C. Although the temperatures of the tail, foot, and nose were within this range little feeding occurred. Dermal temperature does not seem to be a determining factor in fly feeding site preference.

Chaniotis (1975) reported that suckling mice were the least satisfactory source of blood meals for L. trapidoi (Fairchild and Hertig), a species which feeds on a wide variety of mammalian hosts. Gemetchu (1976) reported that P. longipes (Parrot and Martin) which normally feeds on humans would not feed on suckling mice. Although L. anthophora feeds readily on adults of all rodent species offered, it would not feed on suckling mice (SM). When the SM were placed in the feeding cage their body temperature rapidly declined. In an effort

Table 2-6. Temperature (°C) of body regions of anesthetized and non-anesthetized hosts for L. anthophora in laboratory culture.

Animal	Ear			Tail		Rear Foot	Nose	Back
	Tip	Middle	Base	Tip	Base			
Neotoma micropus (anesthetized)	31.5	31.1	34.2	32.4	32.4	33.1	29.7	37.0
Neotoma micropus (non-anesthetized)	31.3	32.3	37.1	31.3	36.8		30.0	37.8
Mesocricetus auretus (anesthetized)	27.7	30.3	34.1	33.2	35.0	35.4	27.8	36.9
Mesocricetus auretus (non-anesthetized)	32.0	32.9	37.2	34.2	35.6	36.2	27.7	36.9
Oryctolagus cuniculus (non-anesthetized)	35.0	36.8					31.4	31.5

to induce sandfly feeding the SM were placed on a cotton pad on a variable temperature plate. Flies were released into a specimen container over the SM. During a 6 hr period the temperature was raised from 26°C (ambient) to 40°C then returned to 26°C. Of 30, 3 day old adults none fed. A thermistor was taped to the SM to insure the skin temperature was the activation source for the heater.

The reason for the failure of L. anthophora to feed on suckling mice remains unknown.

Part of the stimulus for inducing feeding seems not to be the temperature of the host but rather the differential between host temperature and ambient temperature.

Feeding: Behavior

Within 2-5 min after initiating feeding, L. anthophora females fed to repletion (Figure 2-8). The time required for feeding did not change significantly due to host differences.

However, in a few instances it was noted that the flies failed to withdraw their mouthparts from the ear of the host. This phenomenon was observed only with Neotoma and Peromyscus which had been fed upon repeatedly (Figure 2-9). It may be due to the development of a host immune response to sandfly salivary products which prevented the fly from withdrawing its proboscis. This phenomenon has been the subject of considerable investigation with Ornithodorus coriaceus (Theresa Haslett and Michel Laviopierre, personal communication, 1981).

Diuresis to reduce excess water and concentrate the blood meal has been observed in L. anthophora (Figure 2-10) as the excretion of clear

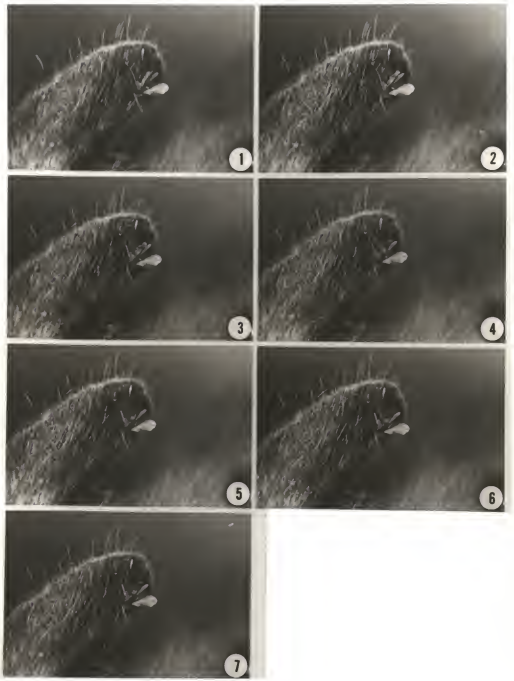


Figure 2-8. Time sequence (20 sec) of L. anthophora feeding on hamster (Mesocricetus auretus) ear.

Figure 2-9. L. anthophora with mouthparts stuck in the ear of (Peromyscus leucopus), white-footed mouse.

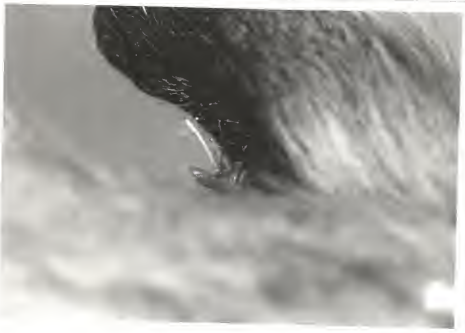


Figure 2-10. L. anthophora excreting clear fluid droplets while feeding.

fluid droplets from the anus while feeding. Chaniotis (1967), Gemetchu (1976), and others have also observed this phenomenon in Phlebotomines.

Feeding: Lymph

Regardless of the host, 3.2-5.6% of the sandflies in a given cohort engorged with a clear fluid that is presumably serum or lymph (Figure 2-11). This behavior indicates that some individuals may have the ability to filter erythrocytes from the blood while feeding or else they may feed by chance from lymphatic capillaries. The latter explanation is feasible since sandflies are telmophages (Lewis 1975) and lymphatic capillaries are numerous in the dermis. Ready (1978) noted that L. longipalpis (Lutz & Neiva) was a non-selective feeder and would feed to engorgement on isotonic saline and whole blood with equal avidity. This may also be true of L. anthophora. Of the 20 serum fed individuals studied in 3 generations 93% did not lay eggs and the maximum number of eggs laid per female was 12 of those that did. In contrast the mean egg production for bloodfed females from the same generations was 30.5. The erythrocyte blood fraction apparently contains nutrients essential for egg production. Ready (1979) found that the concentration of protein ingested had a significant direct relationship to the number of oocytes produced and that the red cell fraction was more important than plasma for egg production. The production of 12 oocytes or less by lymph fed L. anthophora contrasts with the conclusion of Adler and Theodor (1926) that plasma alone was essential for P. papatasi to produce eggs.

Figure 2-11. L. anthophora engorged on serum or lymph.

Figure 2-12. Dead female L. anthophora after peritrophic sac rupture.

Refeeding

Investigations on the vector capability of sandflies have been hampered by the failure of females to survive oviposition. Consequently, demonstration of transmission of Leishmania and Phleboviruses by bite has been difficult to establish (Killick-Kendrick 1979). Killick-Kendrick (1979) and Johnson and Hertig (1961) discussed those species which feed more than once in the laboratory.

In the F_6 and F_{10} generations of L. anthophora maintained in laboratory culture, the individuals that laid eggs within a 24 hr period were released into a feeding cage and offered an anesthetized host for a 30-60 min period daily. Of the flies in those respective generations 19.1% and 16.1% took a second blood meal. Four individuals fed 3x in the F_6 generation. These results are consistent with the observations of Schmidt and Schmidt (1965) on P. papatasi.

No experiments were conducted to discover an optimum oviposition site. If this were determined perhaps a much higher percentage of blood fed females would oviposit, survive oviposition, refeed, and repeat the gonotrophic cycle. Most flies that died prior to oviposition retained eggs in the abdomen. It is unlikely that such high mortality occurs at oviposition in wild populations.

Peritrophic Sac Rupture

The term "membrane" when used to describe the lattice-like sac which surrounds the blood meal in hematophagous insects is a biological misnomer since it is not a trilaminate phopholipid/protein membrane. I suggest adoption of the term "peritrophic sac." Romoser and Rothman

(1973), Romoser (1974), Romoser and Cady (1975) described the lattice-like sac in mosquito larvae, pupae, and adults. The similar structure of the sandfly peritrophic membrane was investigated in detail by Gemetchu (1974). He states that it is formed within 30 min after a blood meal is taken and breaks up about 3 days later.

A phenomenon that occurred consistently in each generation was the apparent rupture of the peritrophic sac and the midgut epithelium. Blood from the gut penetrated all parts of the insect including the thorax, legs, and antennae (Figure 2-12). The specimens that were killed by this phenomenon possibly died as a result of changes in the hemolymph osmoticum and the release of digestive enzymes. In 3 generations (F_8, F_9, F_{10}) the frequency of "peritrophic sac rupture" occurred in 9.9%, 6.6%, and 8.7% of the bloodfed sandflies, respectively. Death of the flies followed feeding in 1-4 days with 75% dead in less than 24 hr.

This phenomenon is not limited to L. anthophora since I have also observed it in Ornithodorus turicata, Ornithodorus dugesi, Triatoma gerstaeckeri, Triatoma sanguisuga, Triatoma neotomae, and Lutzomyia diabolica. The mechanism of gut integrity disruption remains an enigma and does not seem related to the mammalian blood source.

Productivity

Egg production for 12 of 15 generations of L. anthophora reared to date are presented in Table 2-7. The preoviposition period indicated in the table represents the time from blood meal ingestion to egg laying, i.e., the period required for egg development. Since sandflies often extrude 1-3 infertile eggs at death only those females

which laid four or more eggs were considered to have oviposited. No autogeny was observed with this species nor could it be induced by feeding mated females only water or sugar solutions. Egg laying was usually completed in less than 24 hr but could require 2-3 days. Additional blood meals are required for females to lay subsequent batches of eggs.

The first 3 generations were held at 24°C and subsequent generations were held at 28°C with no apparent effect upon egg production. Varying photoperiods also had no apparent effect on egg production or fertility. The effect of blood meal source on fecundity was not investigated.

The number of females which did not take a blood meal was studied in the F_{15} generation and observed to represent 50.3% of the total number eclosed. Those females plus the 19.8% blood fed females which did not lay eggs indicate that 58.5% of the total number of females in a given generation are non-productive. Despite the number of non-productive females, colony numbers could easily be increased to yield as many insects as required for experimentation.

A phenomenon that was consistently observed in each generation was the tendency for those females eclosing in the first half of the generation cycle to lay the majority of the eggs and for many of those emerging in the second half of the generation cycle to die without ovipositing.

An attempt to maintain adults at 32°C proved unsatisfactory since of 36 females produced at that temperature, 11 fed on a mouse (69.4% did not feed), 4/11 (36.4%) laid eggs, and 3/11 (27.3%) fed on serum (no eggs). The mean number of eggs per female was 13.8 ± 9.8. The number of eggs laid by adults held at 32°C was greatly reduced compared to those held at 24°C and 28°C (Table 2-7).

Table 2-7. Fecundity, percent of bloodfed females that laid no eggs, and preoviposition period (days) for 12 generations of L. anthophora reared at 24°C and 28°C, 90% RH.

Generation	n	Fecundity			% Bloodfed Females Laying No Eggs	Preoviposition Period (Days)		
		$\bar{x}$	S	Maximum #		$\bar{x}$	S	Range
F_1	25	42.0	20.5	71	28.0	5.2	1.4	3-11
F_2	99	34.7	21.6	79	53.5	5.5	1.3	4-7
F_3	171	31.7	16.9	64	66.2	7.1	2.0	4-16
F_4	80	39.5	19.0	73	53.3	5.3	1.1	4-8
F_5	61	43.2	13.1	65	43.3	6.4	1.8	4-10
F_6	94	40.6	23.2	107	50.0	6.2	1.3	4-10
F_7	124	36.1	14.3	68	65.3	5.6	0.8	4-6
F_8	121	32.2	18.3	75	45.6	4.5	1.7	2-9
F_9	61	32.9	16.5	63	27.9	4.8	1.0	4-8
F_{10}	92	32.5	17.8	73	32.6	5.8	1.5	2-9
F_{11}	190	26.1	13.1	56	21.8	6.2	1.5	3-11
F_{15}	125	28.0	13.2	56	19.8	6.2	1.8	3-11
Overall Means	1203	35.0	17.3	70.8	38.5	5.7	1.3	2-11

Longevity

Adult longevity for males and non-bloodfed females was determined by holding sandflies individually and placing either distilled water or 30% honey solution on the screen lid. Results are presented in Table 2-8. Sugar-fed adults lived 40-45% longer than those fed on distilled water only.

These results agree with the work of Nayar and Sauerman (1975a,b) and Edmund Davis (personal communication, 1981) who showed that sugar feeding increased longevity of several mosquito species and Culicoides mississippiensis (Hoffman), respectively.

Table 2-8. Comparison of longevity (days) of L. anthophora males and females fed on either distilled water or 30% honey solution at 24°C, 90% RH.

Sex	30% Honey Solution		Distilled H_2O	
	$\bar{x}$	S	$\bar{x}$	S
Female	10.4 (31)*	2.9	7.7 (18)	1.6
Male	10.1 (32)	3.1	7.1 (16)	1.5
Total	10.2 (63)	3.0	7.4 (34)	1.6

*( ) indicates number of individuals tested.

SECTION III

COLONIZATION AND LABORATORY BIOLOGY OF THE SANDFLY, Lutzomyia diabolica (HALL) (DIPTERA: PSYCHODIDAE)

Introduction and Literature Review

Lutzomyia diabolica (Hall 1936) has long been recognized as a pest in South Central Texas (Parman 1919, Lindquist 1936) where it bites humans in and near human dwellings. The status of the species was questioned by Disney (1968) but has been recently resolved by Young and Perkins (1982). Lindquist (1936) studied the life cycle of the species, described the immature stages but did not establish a laboratory colony. Addis (1945a) made an unsuccessful attempt at colonization. Parman (1919) described the bite on humans in detail and suggested that L. diabolica may be a vector of a transient febrile human illness.

Several cases of autochthonous leishmaniasis have been recorded from Texas (Shaw et al. 1976, Simpson et al. 1968, Stewart and Pilcher 1945, and Anderson et al. 1980). Since L. diabolica is the only known man-biting sandfly in the region it is highly suspect as a potential vector.

Field Collection

Although L. diabolica was first taken from Uvalde, Texas, it is widely distributed in Northern Mexico (Najera 1971) and Texas (P.V. Perkins and D.G. Young, personal communication, 1982). For establishment

of a laboratory colony, I collected specimens in July 1981 at Garner State Park, a site located approximately 50 km N of Uvalde, Uvalde Co., Texas, in the Frio river valley near the eastern edge of the Edwards Plateau. The habitat is characterized by open grassland interspersed with oak, acacia, and cedar trees surrounded by rocky hills (Figure 3-1).

All specimens were taken with a tube aspirator and held in field collection vials. The first specimen was taken while feeding on a clerk in the park office at 1800 hrs July 7, 1981. No sandflies were captured with four CDC Light traps set 1 m above ground level in protected areas along 0.5 km of the Frio river for 1 night, and within 20 m of the buildings from which sandflies were taken for a second night. Other workers have taken L. diabolica in CDC Light traps (D.G. Young, personal communication).

Meteorological conditions for the night of 7 July 1981 were 100% overcast with intermittent rain, 25°C, with winds gusting to 15 knots. I noticed L. diabolica feeding on my arms at 2200 hrs while sitting near a light in an open shower/latrine building in an open area with a few adjacent acacia trees. About 40 females were aspirated from the walls of the building between 2300-0300 hrs with the majority taken on the leeward side. The flies were strong fliers and aggressive biters.

The following day I searched 12 latrine/shower buildings in the park, 7 in open, grassy areas and 5 near the river under large trees. Those buildings near the river yielded no sandflies. The other 7 buildings yielded about 100 females and 5 males resting on the walls. No flies were observed flying or feeding during the day. The night of 8 July was humid, 23°C, partly cloudy with 1/4 moon, and winds gusting to 15 knots. Collections made between 2100 hrs (onset of darkness) and 0500 hrs produced about 60 females. About 50 flies were captured

Figure 3-1. Habitat of Lutzomyia diabolica.

Figure 3-2. L. diabolica feeding on a human arm.

9 July in the same buildings as on the 8th but in lesser numbers. L. diabolica appears to be strongly attracted to light especially on warm, humid, overcast nights.

Approximately 10% of the 250 females collected were engorged with blood. All of the unfed flies were allowed to feed on my forearms through the screen lid of the container on 9, 10 July. More than 50% avidly took a blood meal.

For air shipment the plaster in the collecting containers was dampened and vials were packed in a sealed plastic container with wet towels. When received 8 hrs later 90% of the females had died after being trapped in condensation on the sides of the containers. Therefore, specimens shipped by air should be shipped in vials with the plaster dry and wrapped in slightly damp towels.

Feeding

Although Lindquist (1936) reported L. diabolica feeding on humans between 2000 hrs and 2400 hrs it will feed during all hours of darkness. When a cage of flies is held against the skin of a host, females will feed irrespective of light conditions.

When exposed to anesthetized hosts in a feeding cage or while holding a feeding container against the host skin, L. diabolica fed on the following animals: Homo sapiens (human; Figure 3-2), Canis familiaris (dog), Neotoma micropus (woodrat), Mesocricetus auretis (Syrian hamster), Sciurus carolinensis (grey squirrel), Oryctolagus cuniculus (domestic rabbit), Didelphis marsupialis (opossum), Bos taurus (calf), and Equus caballus (horse). Only one unsuccessful

attempt was made to feed flies on Ovis aries (sheep). L. diabolica feeds not only on the ears of mammalian hosts as does L. anthophora but also on the nose, around the eyes, or any other hairless or nearly hairless areas.

Females feed within 24 hrs of eclosion. Feeding behavior is consistent with observations of Lindquist (1936).

Two of 10 females in a feeding cage took a bloodmeal from the inguinal region of a dog that had been infected with Leishmania donovani infantum at least 10 months earlier. Five days after feeding each female had 150-200 promastigotes in the midgut.

Mating

Mating was observed under a wide range of light conditions and before, after, and during feeding.

Egg Hatch and Fertility

No autogeny was observed in this species. Data on fecundity, preoviposition period, and mortality factors are presented in Table 3-1.

First instar larvae do not exhibit synchronous egg hatching in contrast to L. anthophora in which all the eggs of a single batch will hatch within a 2 day period regardless of temperature. As many as 70% of eggs laid by a single L. diabolica female often fail to hatch within a 30 day period whereas nearly all the eggs laid by a single L. anthophora female will hatch. The mechanism of this "partial fertility" phenomenon of some L. diabolica eggs remains a mystery. Lindquist (1936) noted an apparent diapause in the egg stage of L. diabolica from October to

March. The failure of eggs to hatch when laid by a single female was observed from July to December at 20°C, 24°C, 28°C, and 30°C.

Development of individual immatures at various temperatures was not studied because of insufficient numbers resulting from "partial fertility."

I have observed that the egg-adult development time of L. diabolica is 3-6 days less than the 36 days required for L. anthophora at 28°C.

Table 3-1. Lutzomyia diabolica--Fecundity, preoviposition period (days), and mortality factors for 3 generations in laboratory culture at 28°C, 90% RH.

Genera-tion	n	% Bloodfed Females Laying No Eggs	Fecundity			Preoviposition Period (Days)			% Females Peritrophic Sac Rupture	% Females Fed on Serum
			$\bar{x}$	S	Maximum #	$\bar{x}$	S	Range		
1	27	59.3	39.6	11.2	58	5.7	1.7	3-8	11.1	0.0
2	21	28.6	32.0	14.0	51	5.5	1.6	3-9	4.8	4.8
3	51	58.8	36.2	16.0	64	6.2	2.3	3-12		

SECTION IV

TRANSOVARIAN TRANSMISSION OF RIO GRANDE VIRUS BY *Lutzomyia anthophora* (ADDIS) (DIPTERA: PSYCHODIDAE)

Introduction and Literature Review

The genus Phlebovirus of the family Bunyaviridae (Bishop et al. 1980) includes more than 40 viruses (R.B. Tesh, personal communication) distributed over 5 continents (Berge 1975, Karabatsos 1978). Calisher et al. (1977) described Rio Grande virus from isolates made from woodrats, *Neotoma micropus*, collected near Brownsville, Texas, in 1973-1974. *L. anthophora* was suspected of transmitting this virus because of its intimate association with woodrats (Young 1972), the high antibody prevalence (46.3%) of the woodrats (Calisher et al. 1977), and the fact that sandflies transmit other related phleboviruses. Transovarian transmission of phleboviruses by sandflies has been suggested as a mechanism of viral survival (Tesh and Chaniotis 1975). In the present study experiments were undertaken to demonstrate transmission of Rio Grande virus by *L. anthophora* transovarially and by bite.

Materials and Methods

Sandflies

The *L. anthophora* used in these experiments were from the F_7 generation of a closed colony started from stock collected E and NE

of Brownsville, Texas, in May and June 1980 (Section II). Flies used in the experiments were held at 25°C, 80% RH, and a 14:10, light:dark photoperiod regime.

Virus

Rio Grande virus (Strain TBM4-719) was kindly supplied by Dr. Robert Tesh, Yale Arbovirus Research Unit (YARU).

Infection of Sandflies

One hundred twenty, 1-4 day old female sandflies, anesthetized with CO_2 and held on ice, were injected intrathoracically with 10^5 PFU*/ml virus in phosphate-buffered saline by the method of Rosen and Guebler (1974). Maintenance medium (88% Leibowitz medium, 10% tryptose-phosphate broth, 2% heat inactivated fetal calf serum, 1% penicillin 100 units/ml-streptomycin 100 µg/ml) was changed every 4 days. Tubes were examined at regular intervals. After 14 days incubation at 37°C those cultures showing viral cytopathic effect (Tesh et al. 1974) were recorded as positive and discarded. Virus titers were calculated by the method of Reed and Muench (1938). Blood samples were diluted and inoculated into Vero cell tube cultures as above.

Fluid medium from half the positive tubes was tested by complement fixation (Hawkes 1979) to confirm the presence of Rio Grande Virus (RGV) antigen.

Eggs laid by infected flies were reared to adults then tested in the manner described except that a single dilution (1:5) of sandfly suspension was cultured.

*PFU: plaque forming unit.

Suckling mice (Suisse variety) from two litters were inoculated with 0.2 ml 10^5 PFU/ml RGV. One litter was inoculated subcutaneously and the other was inoculated intracerebrally. One mouse from each treatment was bled daily from the carotid artery. All blood samples, 0.1 ml, were diluted with 1.0 ml PBS with 0.05% gelatin and frozen at -70°C for titration.

Virus Assay

Individual flies were triturated in 1.0 ml of dilutent in a sterile 2 ml Ten Broeck tissue grinder. The diluent was phosphate-buffered saline, pH 7.2, containing 0.5% gelatin and 30% heat inactivated bovine serum. Sandfly suspensions were centrifuged at 10,000 rpm for 30 min. The supernatant was prepared in serial ten-fold dilutions from 10^{-1} to 10^{-6}. Four tube cultures of Vero cells were then inoculated with 0.1 ml of each dilution and incubated. Daily samples of 5 infected flies were frozen at -70°C for virus titration to determine the growth of Rio Grande virus (RGV) in the sandflies. Surviving females were offered an anesthetized hamster daily for a 60 min period of feeding. After feeding, engorged females were held at 25°C, 80% RH, in individual oviposition containers.

Fourth instar larvae and pupae were inoculated with RGV intra-abdominally by the same method as the adults.

Suckling mouse blood was tested only at 1:10 dilution.

Infection of Rodents

To determine the fate of RGV in the natural hosts two, white-footed mice, Peromyscus leucopus, and one female Neotoma micropus derived from

stock collected near Brownsville, Texas, were inoculated subcutaneously on the lower ventral abdomen with 10^4 PFU/ml RGV. Daily blood samples were collected from each animal from the retro-orbital capillaries for 7 days.

Results

The growth of RGV in adult female sandflies is shown in Table 4-1. No lag phase occurred during viral replication in the sandfly. The RGV titer in the sandfly increased from Day 1 to Day 7 post inoculation, then declined slightly to an equilibrium that persisted for the life of the fly. The persistent virus titer in the sandfly, 4.2-4.8 $TCID_{50}$*, is similar to those obtained by Jennings and Boorman (1980) with Pacui virus in L. longipalpis and slightly higher than those found by Tesh (1975) when he found 8 phleboviruses replicated in Ae. albopictus and C. fatigans.

Ten 4th instar larvae inoculated with RGV pupated 2 days after inoculation. Within 2 days after pupation all were dead based on the criteria of a) obvious deformity or b) no movement in response to being touched. Ten pupae similarly infected with RGV died within 2 days after inoculation.

The development of RGV in woodrats and white-footed mice is shown in Table 4-2. Although circulating titers were not determined for the infected animals it is clear that the viremia was transient, lasting only 1-2 days and was probably of a low titer.

Attempts to demonstrate transmission of RGV by the bite of infected L. anthophora on uninfected suckling mice were unsuccessful

*Tissue culture infective dose.

because the sandflies refused to feed on the mice (although they subsequently fed on a hamster).

The following mortality was observed after 120 female sandflies were inoculated with RGV: Day 1(18/120, Day 2 (26/102), and Day 3 (7/94). This mortality is attributable to the inoculation procedure.

Twenty-two (22) infected sandflies fed on a hamster and 6 of the 22 (27.3%) survived to oviposit. Based on observations on other generations of L. anthophora the expected survival would have been 61.5%. The inoculation procedure may have caused this reduction. Mean egg production for the 6 flies that oviposited was 28.3 (range 12-47). The mean number of eggs produced does not seem affected by inoculation procedures.

From 170 eggs laid by infected females, 62 adult F_1 progeny were produced with a sex ratio of 1:1. The transovarian transmission (TOT) rate was 54.8% (34 of 62 adults were infected with RGV). The infection rates for males and females was similar, 15/30 (50.0%) and 19/32 (59.3%), respectively. Filial infection rates for the F_1 progeny were not calculated because of insufficient numbers. Each of the 6 infected parents produced 1 or more infected progeny. One adult female survived the F_2 generation and laid eggs but was not infected.

The suckling mice inoculated intracerebrally (IC) or subcutaneously (SQ) with RGV died at 4 and 3 days, respectively. Daily blood samples from a mouse in each group were all positive for Rio Grande virus titrated at 1:10 dilution. The rapid kill rate for the group infected SQ was suspicious because of possible mouse colony contamination with mouse hepatitis virus and the surprising fact that they died before those mice inoculated IC. The experiment was repeated with the same results.

Table 4-1. Growth of Rio Grande virus in L. anthophora after intrathoracic inoculation.

Day Post-Inoculation	Number/Number Infected/Sampled	Range of Titers in Infected Flies*	Mean Titer in Infected Flies*
0 (immediately after inoculation)	5/5	$<10^{0.4}$-$10^{1.1}$	$10^{0.6}$
1	5/5	$10^{0.7}$-$10^{1.7}$	$10^{1.3}$
2	5/5	$10^{1.7}$-$10^{3.4}$	$10^{2.5}$
3	5/5	$10^{1.7}$-$10^{3.7}$	$10^{2.6}$
4	4/5	$10^{2.9}$-$10^{3.1}$	$10^{3.1}$
5	4/5	$10^{3.4}$-$10^{5.0}$	$10^{4.1}$
6	-	-	-
7	5/5	$10^{4.3}$-$10^{5.7}$	$10^{5.0}$
8	2/2	$10^{4.0}$-$10^{4.3}$	$10^{4.2}$
9	2/2	$10^{4.5}$	$10^{4.5}$
10	1/2	$10^{4.8}$	$10^{4.8}$

*Tissue culture infectious $dose_{50}$ per insect.

Table 4-2. Presence of Rio Grande virus in (1) Neotoma micropus and (2) Peromyscus leucopus bled daily for 7 days after subcutaneous inoculation.

Animal	Days Post Inoculation						
	1	2	3	4	5	6	7
Neotoma micropus	0	+	+	0	0	0	0
Peromyscus leucopus							
(a)	0	0	+	0	0	0	0
(b)	0	+	+	0	0	0	0

Discussion

The existence of a transovarian transmission cycle for the Phlebotomus group viruses has long been suspected (Doerr et al. 1909, Whittingham 1924). In the absence of virus isolations, Russian workers (Moshkovski 1937, Petricheva and Alymov 1938) demonstrated transmission of an etiologic agent for Papataci fever through the eggs of P. papataci and demonstrated overwintering of the agent in F_1 larvae of the same species. The recovery of phleboviruses from wild caught male sandflies by several workers (Schmidt et al. 1971, Aitken et al. 1975, Tesh et al. 1974, 1977) lends further evidence to the existence of transovarian transmission in this group. Transovarian transmission of another virus serogroup transmitted by sandflies, Vesicular Stomatis Virus, has been demonstrated by Tesh et al. (1972).

Despite overwhelming evidence indicating that Papataci fever virus and other phleboviruses are transmitted by phlebotomine sandflies (Schmidt 1971, Tesh 1975), no quantitative laboratory studies have been conducted to demonstrate transmission by bite or by transovarial means. The lack of controlled experiments has been partially a function of the difficulty encountered in rearing sandflies.

In this series of experiments we were unable to demonstrate transmission by bite due to the lack of susceptible laboratory animals, by the fact that L. anthophora refused to feed on suckling mice, and a lack of knowledge on virus dynamics in a rodent host. Therefore, infection was established by means of intrathoracic injection. Sandflies infected by injection transmitted Rio Grande virus to 54.8% of their progeny. This is the first demonstration of transovarian transmission of a Phlebovirus by sandflies.

Calisher et al. (1977) found neutralizing antibody to Rio Grande virus in woodrats, opossums, gopher tortoises, horses, several species of small rodents, birds, and a horned toad. I was unable to induce L. anthophora to feed on gopher tortoises, or horses. This suggests that L. anthophora probably is not the only arthropod vector of Rio Grande virus. Other hematophagous arthropods that I recovered from the woodrat nests were Ornithodorus dugesi (often identified as O. tulaje), Triatoma gerstaeckeri, Triatoma sanguisuga, and Triatoma neotomae. Johnson (1966) also reported several species of fleas and Ixodidae from the woodrat nests. It is highly likely that mosquitoes also use the nests for resting sites. Some of these other arthropods are catholic in their feeding behavior and could possibly transmit Rio Grande virus to animals not fed on by L. anthophora, and may be capable of transovarian transmission.

The ecology of Rio Grande virus remains to be thoroughly studied. Although Neotoma micropus and Peromyscus leucopus are susceptible to infection by subcutaneous inoculation the infection is transient. McLean et al. (1982) confirmed the results in Table 4-2 in that the viremia in woodrats is short-lived, 2.5 days, and of low titer, mean 3.65 $\log_{10}$ PFU/ml in Vero cell culture. As yet, oral infection of L. anthophora has not been demonstrated nor is the minimum infective oral dose known. McLean et al. (1982) also determined that nearly all the woodrats developed neutralizing antibody thus becoming refractory to infection for life. This indicates that only young woodrats are likely to be susceptible to infection after maternal antibody is no longer present and they could only serve as amplifying hosts for 2.5 days during their lifetime. The high transovarian transmission rate

obtained with L. anthophora could account for maintenance of RGV in nature by the stabilization mechanism discussed by Tesh and Shroyer (1980).

At Uvalde, Texas, Parman (1919) reported an epidemic of a mild febrile illness (102-104°F, 3 days duration) concurrent with large populations of L. diabolica, a man-biting species also known to feed on woodrats, opossums, cattle, dogs, and horses. Parman (1919) thought the possible association of the sandfly numbers and the epidemic was a suspicious coincidence requiring investigation. The symptoms described by Parman (1919) are consistent with those known to occur after infection by phleboviruses (Bartonnelli and Tesh 1976, Tesh et al. 1977).

From this study I must conclude that L. anthophora is probably important in maintaining Rio Grande virus in the woodrat population but may not solely account for its transmission. In order to more completely understand the ecology of Rio Grande virus detailed field and laboratory investigations of the vector potential of other arthropods must be undertaken.

SECTION V

RIO GRANDE VIRUS AND Triatoma gerstaeckeri (STAL) (HEMIPTERA: REDUVIIDAE)

Introduction and Literature Review

The hematophagous Hemipterans, the Cimicidae and the Triatominae, have been considered ideal potential vectors of arboviruses because of the large blood meal ingested, their relative longevity, and their cosmopolitan feeding habits. Kitselman and Grundman (1940) reported isolating Western Equine Encephalitis (WEE) virus from Triatoma sanguisuga taken in a Kansas pasture where animals had died of the disease in previous years. Mangiafico et al. (1968) found that 2 species of Triatome, R. prolixus and T. infestans, would harbor WEE virus 14-20 days when unpunctured. When punctured to simulate cannabalistic feeding virus survived 98 days and one bug transmitted the virus by bite. Justines and Sousa (1977) obtained similar results with punctured bugs and bugs infected with Trypanosoma cruzi. Hayes et al. (1977) found the cliff swallow bug, Cimicidae, to be capable of overwintering and transmitting Ft. Morgan virus (Calisher et al. 1980).

In view of the findings noted and realizing that Triatoma gerstaeckeri was known to feed on all of the animals (Lent and Wygodzinsky 1979), in which Calisher et al. (1977) had found neutralizing antibody to Rio Grande virus, the vector potential of T. gerstaeckeri was investigated. Thurman (1945) and Pippin (1970) reported finding

T. gerstaeckeri infected with T. cruzi in Neotoma nests. Pippin (1970) noted 30.1% of the bugs found in the nests were infected.

Materials and Methods

Virus

Rio Grande virus (strain TMB4-719) was kindly supplied by Dr. Robert Tesh, Yale Arbovirus Research Unit. Additional quantities of virus were prepared by passage through suckling mouse brain.

Triatomes

A colony of T. gerstaeckeri was started from specimens collected near Brownsville, Texas, in June, 1980, and augmented with specimens collected near Lake Medina, San Antonio, Texas, in July, 1981. First and second instar nymphs from the colony were used in the transmission experiments.

One hundred forty (140) 2-3 week old first instar nymphs were fed on 3 suckling mice that had been given Rio Grande virus by intracerebral inoculation 4 days earlier. Three other mice from the same litter died on Day 5 post inoculation.

Eight, 16, and 24 days after the initial feeding 80 first instar nymphs that had fed on viremic mice fed on 6 unexposed suckling mice. After 1 week the suckling mice showed no apparent signs of viral infection.

Results and Conclusions

The failure to infect 6 suckling mice fed on by 80 nymphs that had fed on viremic mice 8, 16, or 24 days earlier indicates that T. gerstaeckeri

may not be capable of transmitting Rio Grande virus. However, the additional experiments should be conducted before the Triatominae are proven to be incompetent vectors of phleboviruses. These should include fluorescent antibody localization of viral antigen to determine its fate in the insect.

SECTION VI

PURIFICATION OF RIO GRANDE VIRUS

Introduction

Since Calisher et al. (1977) first characterized Rio Grande virus little other descriptive work has been performed. Because of its ecological relationship to other members of the group, Rio Grande virus was placed in the genus Phlebovirus of the family Bunyaviridae by Bishop et al. (1980). In this section a purification scheme for the virus is described and electron micrographs of the virus are presented.

Materials and Methods

Virus

Rio Grande virus (Strain TMB4-719) was kindly supplied by Dr. Robert Tesh, Yale Arbovirus Research Unit. Additional quantities of virus were produced by intracerebral inoculation of 2-4 day old suckling mice with 0.01-0.02 ml stock virus. Four days after inoculation the mouse brains were harvested and triturated in 2 ml Ten Broeck tissue grinders with 1x sterile phosphate buffered saline (PBS), pH 7.4.

Infection of Cells and Virus Purification

The purification scheme detailed below was based on several others previously used for arboviruses (Kaariainen et al. 1969, Obijeski et al.

1976, Clewley et al. 1977). Four 150 cm^2 tissue culture flasks of Vero cells in a confluent monolayer were inoculated with 6 ml mouse brain suspension and allowed to adsorb for 1 hr at 37°C. Cultures were then overlaid with 50 ml minimum essential medium (MEM) and incubated at 37°C. After 24 hrs the supernatant was poured off, 75 ml MEM was overlaid, and the cells were incubated for an additional 6 days at 37°C after which >90% of the cells were destroyed. Supernatants were collected, frozen to -70°C, thawed to 37°C, then clarified by low-speed centrifugation at 4°C for 30 min at 8,000 g in a Sorvall RCB-2 centrifuge to remove cell debris.

Virus was recovered from the clarified supernatant by precipitation in a 7% polyethylene glycol/0.4 M NaCl solution stirred for 4 hr at 4°C followed by centrifugation at 10,000 g for 20 min. The pellet was resuspended in 4 ml TSE buffer (0.01 M Tris hydrochloride buffer, pH 7.5, containing 0.1 M NaCl and 0.002 M EDTA) and loaded over a combination equilibrium: viscosity gradient of potassium tartrate (McCrea et al. 1961) and glycerol (KT-GLY).

Two 10 ml KT-GLY gradients were made with a Bethesda Research Products Gradient Former® and an LKB peristaltic pump®. Fourteen milliliters of 50% (w/w) potassium tartrate in TSE buffer was loaded into the inside chamber and 16 ml of 30% (w/w) glycerol was loaded into the outside chamber (Obijeski et al. 1974, Barzilai et al. 1972).

Virus suspensions loaded onto KT-GLY gradients were centrifuged in an SW 41 rotor at 4°C for 8 hr at 40,000 g. Three nearly inseparable visible bands were produced. The virus fraction was collected at 254 nm (RNA absorbance peak) using an ISCO gradient column fractionator and flow densitometer®.

Electron Microscopy

A drop of virus suspension was placed on a Formvar carbon coated grid and allowed to dry for 1 min before the excess fluid was wicked off with filter paper. The grid was then negatively stained for 45 sec with 2%, pH 6.8, phosphotungstic acid with KOH, using 50 µg/ml Bacitracin as a spreading agent (Gregory and Pirie 1973). Specimens were examined at 75 KV, 50,000x and 100,000x in a Hitachi 600 Transmission Electron Microscope.

Results

Electron micrographs (Figure 6-1) show that the virion is spherical and possesses an envelope bearing small spikes.

The virion is 71 nm in diameter as determined using a reference catalase crystal. This is within the size range of 60-90 nm that is characteristic of the Bunyaviridae (Bishop et al. 1980).

Discussion

The size and morphology of the Rio Grande virion is consistent with those described for the genus Phlebovirus (Bishop et al. 1980).

An additional purification step of centrifuging the virus suspension in a 20-70% (w/v) sucrose gradient at 4°C and 35,000 g for 4 hr was not used since it was possible to recover the virus after the equilibrium-density centrifugation in potassium tartrate.

In order to verify that the virions shown in the electron micrographs retained infectivity, 1 ml of virus suspension was adsorbed onto

Figure 6-1. Electron micrographs of purified Rio Grande virus at 125,000x.

a confluent monolayer of Vero cells in a 75 cm^3 tissue culture flask which was then overlaid with 50 ml minimum essential medium. After 6 days incubation at 37°C nearly 100% of the cells were destroyed.

SECTION VII

A COMPARISON OF OOCYTE TOPOGRAPHY OF FIVE PHLEBOTOMINE SANDFLIES (Lutzomyia) WITH THE SCANNING ELECTRON MICROSCOPE (DIPTERA: PSYCHODIDAE)

Introduction

The egg surface structure of 19 neotropical Phlebotomine species has been described (Zimmerman et al. 1977, Ward and Ready 1975) using the scanning electron microscope (SEM). Ward and Ready (1975) noted three species-specific topographic patterns, i.e., polygonal, parallel ridging, and volcano-like. Several authors (Chaniotis and Anderson 1964, Addis 1945,Lindquist 1936, Barreto 1941, and Sherlock 1957a,b, 1963) described and figured the eggs of 17 neotropical sandfly species using light microscopy. After examining the literature cited and eggs from the 5 species described herein we propose adding another category to the patterns of Ward and Ready (1977); that is, parallel ridges connected or parallel ridges unconnected.

The classification of eggs of 41 species of New World sandflies according to the proposed scheme is presented in Table 7-1.

Materials and Methods

Eggs were obtained from females reared in laboratory colonies. The preparation method of eggs for SEM based on the work of Quattlebaum and Carner (1980) is as follows:

Table 7-1. Classification of 41 species of Neotropical phlebotomine sandfly eggs based on oocyte topographic patterns.

Describer	Topographic Pattern: Polygon	Parallel Ridges (connected)	Parallel Ridges (unconnected)	Volcano-Like
Endris et al.	L. texana L. vexator L. shannoni	L. cruciata spp. L. anthophora	L. diabolica	
Sherlock	L. lenti L. bahiensis		L. renei	
Zimmerman et al.	L. sanguinaria L. trapidoi L. ylephilator L. gomezi			
Chaniotis	L. vexator occidentalis			
Ward and Ready	L. antanesi L. yuilli L. nsp. 260.43 L. nsp. 260.44 L. dendrophyla L. gomesi		L. longipalpis	L. flaviscutellata L. complexa L. lainsoni L. carrerai L. davisi L. paraensis
Barreto	L. guimaraisi L. pessoai L. fischeri L. limai L. monticola	L. pestanai L. arthuri L. intermedia	L. lanei L. whitmani L. alphabetica	

1. Eggs were placed on a filter paper disc in a 1 cm deep plastic container cut from a plastic film cannister.
2. The plastic container was floated in a 50 ml Tri-pour® polystyrene beaker containing 5 ml aqueous 1% OsO_4
3. The paper lid was installed and the entire container was sealed in Parafilm® and held in an exhaust hood at room temperature for 5 days.
4. After 5 days exposure to osmium vapor the inner container was transferred to a covered petri dish for 24 hr to allow slow drying of the eggs.
5. Eggs were attached to an SEM stub using either double-sided tape or 0.1% aqueous hydrobromide polylysine (Polysciences, Inc., Warrington, PA 18976), sputter-coated with approximately 300 Å of gold in an Eiko Engineering IB-2 Ion Coater, and examined in a Hitachi S-450 scanning electron microscope (SEM) at 20 KV.

Eggs were measured in microns at 100x with a compound microscope and an ocular micrometer. Intact fresh eggs or recently hatched eggs were acceptable whereas old eggs or infertile eggs usually collapsed making accurate measurement difficult. Eggs to be measured were placed on a microscope slide in Histocon®. In each sample the eggs were produced by 5-10 females.

Results

The SEM micrographs of the sandfly eggs are shown in Figures 7-1 and 7-2. Descriptions of eggs of each species are as follows. Measurements

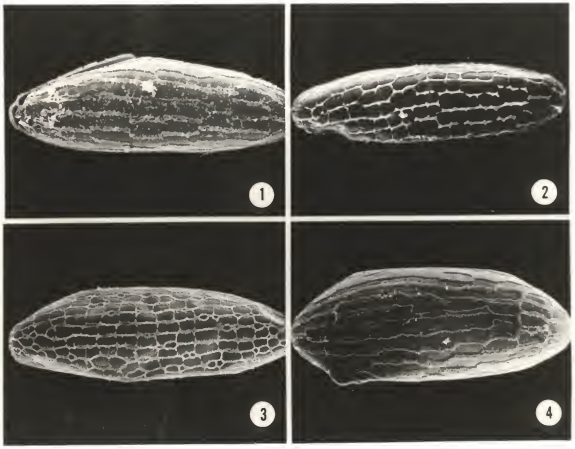


Figure 7-1. Scanning electron micrographs of eggs of four sandfly species. (1) Lutzomyia diabolica, (2) Lutzomyia shannoni, (3) Lutzomyia vexator, (4) Lutzomyia cruciata spp.

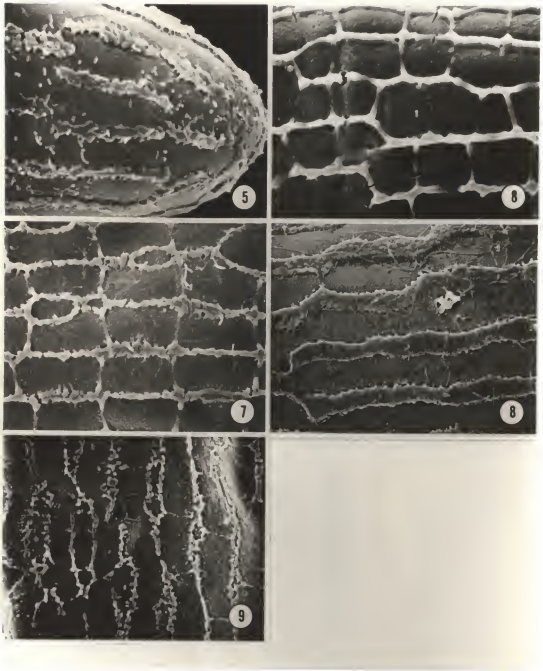


Figure 7-2. Scanning electron micrographs of oocyte topography of five species of sandfly. (5) Lutzomyia diabolica, (6) Lutzomyia shannoni, (7) Lutzomyia vexator, (8) Lutzomyia cruciata spp., (9) Lutzomyia anthophora. 7,000x.

given are the range, mean, and standard deviation for egg length and width for each species.

Lutzomyia shannoni (Dyar, 1929), Florida specimens

Figure 7-1(2), 7-2(6)

Size: N = 102, L: 290-340 (330 ± 10), W: 70-110 (90 ± 10)

Exochorion: High, narrow longitudinal ridges connected by prominent perpendicular ridges forming 4 and 5 sided polygons which are frequently rectangular.

Lutzomyia diabolica (Young and Perkins 1982), Uvalde Co., Texas

Figure 7-1(1), 7-2(5)

Size: N = 47, L: 340-370 (350 ± 10), W: 90-110 (100 ± 10)

Exochorion: Surface topography is characterized by a series of discontinuous parallel longitudinal ridges that are not laterally connected.

Lutzomyia vexator (Coquillett 1907), Levy Co., Florida

Figure 7-1(3), 7-2(7)

Size: N = 193, L: 330-390 (380 ± 10), W: 80-110 (100 ± 10)

Exochorion: Surface topography consists of delicate parallel longitudinal ridges with regular perpendicular connections that form polygons which are nearly square. There are also occasional oblong cells.

Lutzomyia anthophora (Addis 1945), Cameron Co., Texas

Figure 7-2(9)

Size: N = 100, L: 330-370 (340 ± 10), W: 80-100 (80 ± 10)

Exochorion: Reticulation consists of weak parallel longitudinal ridges with slight perpendicular connections at irregular intervals.

Lutzomyia cruciata spp. (Young and Perkins 1982), Alachua Co., Florida

Figure 7-1(4), 7-2(8)

Size: N = 61, L: 320-370 (340 ± 10), W: 80-120 (100 ± 10)

Exochorion: Wide, flat, parallel longitudinal ridges with occasional weaker connecting ridges which are not usually perpendicular to the longitudinal ridges.

Discussion

Several techniques were tried for preserving the eggs to prevent collapse under vacuum in the SEM column. The method used here yielded the best results when fertile eggs were used.

A "standard" EM fixation procedure using 1% OsO_4 as a fixative followed by 5% aqueous acrolein, dehydration in dimethoxypropane and acetone, then critical point drying with Freon as a transition solvent proved unsuccessful because most specimens collapsed in the SEM. Lyypholization and critical point drying of eggs without fixation were also unsuccessful.

A technique which was not used but one which may be promising is freeze drying.

The size variation of eggs laid by individual females was determined by measuring 10 eggs from each of 10 L. vexator females. The variation in egg length and width between females ranged from 10-50 microns and from 10-30 microns, respectively. As a result of broad intraspecific variation it is not possible to separate the eggs of different sandfly species by size. Therefore the surface sculpturing is the only characteristic of the egg that can be used for species determination.

SECTION VIII

PHOTOGRAPHIC TECHNIQUES

Quality photographs of living sandflies, Culicoides, and other small insects have been notably lacking from the literature due to the difficulty of producing them. Part of the difficulty involved in photographing these insects is containing them. Two types of specialized containers were developed in order to photograph sandflies.

The first container (Figure 8-1) was constructed from a 40 liter aquarium. Two sides were replaced with 2 mm Plexiglas. Three access ports (19 x 19 cm) were cut in the sides and fitted with Plexiglas compression frames (2 cm wide x 64 mm thick). Sleeves 50 cm in length made of 15 cm surgical stockinet (Johnson & Johnson) were secured around the ports by the compression frames which were attached with 8 brass screws (10/24 x 1") and wing nuts. The screws and flat washers wereglued in place with epoxy glue for ease of attaching the sleeves. Three sleeves are required, 1 for the camera, 1 for manipulating specimens, and 1 for the host arm. A Plexiglas insertwas attached to the top frame of the aquarium with silicone glue thus making a removable top. The back and bottom of the aquarium is covered with a 1 cm layer of plaster of Paris to provide a light reflective background.

The second type of photographic chamber (Figure 8-2) was constructed from a spectrophotometric cuvette (Wallace & Tiernan, Co.,

Figure 8-1. Chamber for photographing hematophagous insects feeding on humans and small mammals.

Figure 8-2. Chamber for photographing small insects.

Belleville, NJ) that is 7.5 x 2.5 x 1.5 cm. It was covered on the bottom and ends with a 1 cm layer of white polyethylene foam (Ward's Scientific Co., Rochester, NY). A Kodak Neutral Test card 90% reflectance on the white side and 18% on the gray side was used for a background behind the cuvette chamber.

A third type of photographic chamber used occasionally was the rectangular adult feeding cage.

All of the sandfly photographs presented in this manuscript were photographed with a 200 mm Medical Nikkor lens at 3x magnification, F45. This lens was used because it has a built-in ring flash and provides 8 cm working distance at 3x magnification. The camera used was a Nikon F2 Photomic with type "C" focusing screen and cable release. Because the lens is of a fixed focal length it was necessary to mount the camera on a Slik 2-axis focusing rail on a tripod in order to achieve reproducible results.

Fujichrome film, ASA 100, was used for all the photographs. Black and white prints were all made from color slides using Ilford XP400 ultra fine grain film for an internegative.

SECTION IX

SUMMARY

During the four year course of this study the following objectives were achieved:

1. Methods for laboratory culture of phlebotomine sandflies were developed. Use of these culture techniques will permit detailed quantitative study of the vector competence of sandflies for viral and other parasitic diseases. To date twelve species have been reared in closed colony for as many as 25 generations using these techniques.

2. Detailed studies on the laboratory biology of two species of Neotropical phlebotomine sandfly, Lutzomyia anthophora and Lutzomyia diabolica were conducted over 16 and 7 generations, respectively.

3. Transovarian transmission of a Phlebovirus by a sandfly was conclusively demonstrated for the first time. Nearly 55% of L. anthophora females injected with Rio Grande virus transmitted it to their progeny.

4. The oocyte surface sculpturing of 5 species of Neotropical sandflies were described for the first time with the scanning electron microscope. The topographic patterns can be used to identify these sandfly species.

5. A method for purification of Rio Grande virus was developed. The virus was purified and photographed in a transmission electron microscope. This is one of the first recorded purifications and characterizations of a Phlebovirus.

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BIOGRAPHICAL SKETCH

On July 31, 1948, Richard G. Endris entered the world at the rural farming community of Pana, Illinois. After twelve years residence there his family moved to Somerville, NJ, where he completed primary school and graduated from Somerville High School in 1966. After graduating from Rutgers University, New Brunswick, NJ, with a degree in animal science he enrolled at the University of Florida for a master's degree in entomology which he completed in 1972. The subsequent three years were spent in the U.S. Army as a medical entomologist with assignments to the 82nd Airborne Division and 8th Army Korea. In 1976 he left the Army to work as a health consultant to Fluor Corporation in Asia for a year before enrolling in a Ph.D. program in entomology at the University of Florida in 1977.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Dr. Stephen G. Zam
Associate Professor of Microbiology and Cell Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1982

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Dean, College of Agriculture

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